August 2012

qBiomarker Copy Number PCR Handbook

qBiomarker Copy Number PCR Array

qBiomarker Copy Number PCR Assay

For real-time PCR-based, copy number alteration and variation analysis

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Array Contents

qBiomarker Copy Number PCR Arrays						
Catalog no.	**337802**					
Number of samples	**2**	**12**	**24**	**4**	**16**	**64**
96-well plates (A, C, D, F formats)	2	12	24	–	–	–
384-well plates (E, G formats)	–	–	–	1	4	16
Rotor-Discs (R format)	2	12	24	–	–	–
Optical Thin-Wall 8-Cap Strips (12 per plate; A, D formats only)	24	144	288	–	–	–
Optical Adhesive Film (1 per plate; C, E, F, G formats only)	2	12	24	1	4	16
Rotor-Disc® Heat Sealing Film (1 per Rotor-Disc; R format only)	2	12	24	–	–	–
384EZLoad Covers (1 set of 4 per plate; E, G formats only)	–	–	–	1 set	4 sets	16 sets
Handbook	1	1	1	1	1	1

Custom qBiomarker Copy Number PCR Arrays				
Catalog no.		**337801**		
Number of samples	**Varies**	**Varies**	**Varies**	**Varies**
96-well plates (A, C, D, F formats)	12	24	–	–
384-well plates (E, G formats)	–	–	6	24
Rotor-Discs (R format)	12	24	–	–
Optical Thin-Wall 8-Cap Strips (12 per plate; A, D formats only)	144	288	–	–
Optical Adhesive Film (1 per plate; C, E, F, G formats only)	12	24	6	24
Rotor-Disc Heat Sealing Film (1 per Rotor-Disc; R format only)	12	24	–	–
Handbook	1	1	1	1

Assay Contents

qBiomarker Copy Number PCR Assays	
Catalog no.	**337812**
Number of samples	**200**
qBiomarker Copy Number PCR Assay; provided in solution	200 µl
Handbook	1

Mastermix Contents

qBiomarker SYBR® Fluor Mastermix*	**(2)**	**(12)**	**(24)**	**(8)**	**(25 ml)**
Catalog no.	**337830**	**337832**	**337833**	**337831**	**337839**
Number of array reactions	**2 x 96-well**	**12 x 96-well**	**24 x 96-well**	**4 x 384-well**	**2000 x 25 µl reactions**
2x Mastermix, containing: HotStart DNA *Taq* Polymerase, PCR Buffer, dNTP mix (dATP, dCTP, dGTP, dTTP), SYBR Green dye, Fluorescein passive reference dye	2 x 1.35 ml	12 x 1.35 ml	24 x 1.35 ml	8 x 1.35 ml	25 ml

* Suitable for use with the following real-time cyclers: Bio-Rad® models iCycler®, iQ™5, MyiQ™, MyiQ2.

qBiomarker SYBR ROX™ Mastermix*	**(2)**	**(12)**	**(24)**	**(8)**	**(25 ml)**
Catalog no.	**337820**	**337822**	**337823**	**337821**	**337829**
Number of array reactions	**2 x 96-well**	**12 x 96-well**	**24 x 96-well**	**4 x 384-well**	**2000 x 25 µl reactions**
2x Mastermix, containing: HotStart DNA *Taq* Polymerase, PCR Buffer, dNTP mix (dATP, dCTP, dGTP, dTTP), SYBR Green dye, ROX passive reference dye	2 x 1.35 ml	12 x 1.35 ml	24 x 1.35 ml	8 x 1.35 ml	25 ml

* Suitable for use with the following real-time cyclers: Applied Biosystems® models 5700, 7000, 7300, 7500 (Standard and Fast), 7700, 7900HT (Standard and Fast 96-well block, 384-well block), StepOnePlus™, ViiA 7 (Standard and Fast 96-well block, 384-well block); Eppendorf® Mastercycler® ep *realplex* models 2, 2S, 4, 4S; Stratagene® models Mx3000P®, Mx3005P®, Mx4000®; Takara TP-800.

qBiomarker SYBR ROX FAST Mastermix*	(2)	(12)	(24)	(8)	(25 ml)
Catalog no.	**337840**	**337842**	**337843**	**337841**	**337849**
Number of array reactions	**2 x 96-well**	**12 x 96-well**	**24 x 96-well**	**4 x 384-well**	**2000 x 25 µl reactions**
2x Mastermix, containing: HotStart DNA *Taq* Polymerase, PCR Buffer, dNTP mix (dATP, dCTP, dGTP, dTTP), SYBR Green dye, ROX passive reference dye	2 x 1.35 ml	12 x 1.35 ml	24 x 1.35 ml	8 x 1.35 ml	25 ml

* Suitable for use with the Applied Biosystems models 7000, 7300, 7500 (Standard and Fast), 7700, 7900HT (Standard and Fast 96-well block, 384-well block), StepOnePlus; ViiA 7 (Standard and Fast 96-well block, 384-well block); Eppendorf Mastercycler ep *realplex* with or without ROX filter set; Stratagene models Mx3000, Mx3500, Mx4000; Takara TP-800; Rotor-Gene® Q (QIAGEN), Rotor-Gene 6000.

Shipping and Storage

qBiomarker Copy Number PCR Arrays are shipped at ambient temperature, on blue ice/cold packs, or on dry ice, depending on the destination and accompanying products. For long-term storage, store qBiomarker Copy Number PCR Arrays at –15°C to –30°C.

qBiomarker Copy Number PCR Assays are shipped at ambient temperature, but must be stored at –20°C on arrival, and at –15°C to –30°C for long-term storage.

qBiomarker SYBR Mastermixes are shipped on blue ice/cold packs. For long-term storage, keep tubes at –20°C. If the entire volume will not be used at once, we recommend dividing into aliquots and storing at –20°C. Avoid repeated freezing and thawing. If stored under these conditions, qBiomarker SYBR Mastermixes are stable for 6 months after receipt.

Intended Use

qBiomarker Copy Number PCR Arrays, qBiomarker Copy Number PCR Assays, and qBiomarker SYBR Mastermixes are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding qBiomarker Copy Number PCR Arrays, qBiomarker Copy Number PCR Assays, qBiomarker SYBR Mastermixes, or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/Support/safety where you can find, view, and print the SDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Quality Control

In accordance with QIAGEN's Quality Management System, each lot of qBiomarker Copy Number PCR Arrays, qBiomarker Copy Number PCR Assays, and qBiomarker SYBR Mastermixes is tested against predetermined specifications to ensure consistent product quality.

Introduction

qBiomarker Copy Number PCR Arrays and Assays are designed for highly sensitive detection and profiling of copy number alterations (CNAs) and copy number variations (CNVs).

CNAs are an important type of genomic aberration in cancer cells. Gene amplifications (which activate oncogenes) and deletions (which inactivate tumor suppressor genes) are important CNAs that affect cancer-related genes, in addition to other genomic changes (such as point mutations, translocations, and inversions). Most of the cancer-related genes affected by CNAs have been defined as key genes in cancer-signaling pathways involved in carcinogenesis and cancer progression.

CNVs are an important source of genetic diversity. De novo, locus-specific mutation rates appear to be much higher for CNVs than for other forms of genetic variation, such as SNPs. CNVs can cause Mendelian diseases through various molecular mechanisms such as gene dosage, gene disruption, gene fusion, and positional effect, and they can also be associated with complex diseases and traits.

CNA and CNV profiling

Quantitative analysis of CNAs or CNVs using qBiomarker Copy Number PCR Arrays is a useful tool for study of the molecular mechanisms of disease and genetic traits, and also provides the potential for discovery of novel biomarkers.

qBiomarker Copy Number PCR Arrays are available to target key CNAs of cancer-related genes that are known to occur in specific tissue types, gene pathways, and functional groups. The genes are selected from peer-reviewed publications based on their frequency, their function in cancer-signaling pathways, and their association with a cancer phenotype or progression. In addition, qBiomarker Copy Number PCR Arrays are available for CNV profiling that focus on key CNVs known to contribute or correlate with selected complex human diseases, genetic diseases, or traits. Each qBiomarker Copy Number PCR Array enables analysis of up to 95 genes or regions of interest (GOIs or ROIs). The arrays are compatible with most real-time PCR platforms.

Copy number assays

qBiomarker Copy Number PCR Assays provide a method for obtaining specific, accurate, reproducible, and easy-to-interpret copy number change results for an individual gene or region of interest (GOI or ROI). Every assay has been bench-verified and is ready-to-use in microarray follow-up studies, specific target screening, and related studies.

Principle and procedure

All qBiomarker Copy Number PCR Assays are designed in unique regions of the genome. A multi-copy reference assay, the qBiomarker Multicopy Reference Copy Number PCR Assay (MRef) is included on each array. The qBiomarker Multicopy Reference Copy Number PCR Assay is also recommended for use with each qBiomarker Copy Number PCR Assay (must be ordered separately, cat. no. VPH000-0000000A). The reference assay recognizes a stable sequence that appears in the human genome over 40 times, and whose copy number is not affected or minimally affected by local genomic changes. Inclusion of this reference assay during testing allows use of the $\Delta\Delta C_T$ method to accurately make copy number calls or relative copy number change calls for specific targets (see "Appendix B: Data Analysis Using the $\Delta\Delta C_T$ Method", page 49).

Each qBiomarker Copy Number PCR Array contains a panel of qBiomarker Copy Number PCR Assays for a set of selected GOIs or ROIs. Four replicates of each assay are included to increase the accuracy of copy number calls using statistical analysis. The arrays are available in 96-well plate, 384-well plate, and Rotor-Disc formats. A 96-well plate array and a Rotor-Disc array each contain 4 replicates of 24 genes (23 target genes plus MRef) for one sample, and a 384-well plate array can either contain 16 replicates of 24 genes (23 target genes plus MRef) for 4 samples, or 4 replicates of 96 genes (95 target genes plus MRef) for one sample. For more details, see page 17.

Description of protocols

The qBiomarker Copy Number PCR procedure (see workflow, page 12) starts with genomic DNA purification. After quality control, the DNA is mixed with the ready-to-use qBiomarker SYBR Mastermix. The mixture is aliquoted into each well of the array plate containing preloaded gene-specific primers. After real-time PCR, the copy-number profile of a particular sample is determined using the $\Delta\Delta C_T$ method, measuring the difference in ΔC_T values (target gene C_T – reference assay C_T) between a test sample genome and a reference genome (see "Appendix B: Data Analysis Using the $\Delta\Delta C_T$ Method", page 49).

The simplicity of the qBiomarker Copy Number PCR Array format and protocol allows routine CNA and CNV profiling in any research laboratory with access to a real-time PCR cycler.

qBiomarker Copy Number PCR workflow overview

Purify DNA using the QIAamp® DNA Mini Kit, DNeasy® Blood & Tissue Kit, or QIAamp DNA FFPE Tissue Kit (approximately 30 minutes).

↓

Measure DNA concentration and purity by UV spectrophotometry.

↓

Optional: Run quality-control check using DNA QC Plate.

↓

Set up and run real-time PCR using qBiomarker Copy Number PCR Arrays or Assays (2 hours).

↓

Analyze data using the online analysis template (10–20 minutes).

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

In addition to the qBiomarker Copy Number PCR Array or Assay and qBiomarker SYBR Mastermix, the following are required:

- For qBiomarker Copy Number PCR Assays: 96-well or 384-well PCR plate with Optical Thin-Wall 8-Cap Strips or Optical Adhesive Film or Rotor-Disc with Rotor-Disc Heat-Sealing Film or PCR tubes
- Purified DNA samples (see "DNA purification", page 16)
- Spectrophotometer*, cuvettes, and nuclease-free Tris·Cl, pH 8.0, for measuring DNA quality and quantity
- Nuclease-free water (e.g., QIAGEN, cat. nos. 129114, 129115, or 129117); do not use water treated with diethylpyrocarbonate (DEPC)
- Real-time PCR cycler* (see Tables 1 and 2, pages 14 and 15)
- Single- and multichannel pipets with nuclease-free tips*
- Nuclease-free microcentrifuge tubes
- Centrifuge* and rotor for PCR array plates
- Crushed ice
- Optional: DNA QC Plate (QIAGEN, please inquire for cat. no.) for assessing DNA quality
- Optional: RT^2 PCR Array Loading Reservoir (cat. no. 338162) to assist in loading PCR plates

* Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

Table 1. Real-time PCR cyclers suitable for use with qBiomarker Copy Number PCR Arrays

Plate format	Plate type	Real-time PCR cyclers
A	96-well	Applied Biosystems models 5700, 7000, 7300, 7500, 7900HT (96-well block), ViiA 7 (96-well block); Bio-Rad/MJ Research Chromo4™, iCycler, iQ5, MyiQ, MyiQ2; Stratagene models Mx3000P, Mx3005P; Eppendorf Mastercycler ep *realplex* models 2, 2S, 4, 4S; TaKaRa TP-800
C	96-well	Applied Biosystems models 7500 Fast (96-well), 7900HT Fast (96-well block), StepOnePlus, ViiA 7 Fast (96-well block)
D	96-well	Bio-Rad CFX96™; Bio-Rad/MJ Research models DNA Engine Opticon®, DNA Engine Opticon 2; Stratagene Mx4000
E	384-well	Applied Biosystems models 7900HT (384-well block), ViiA 7 (384-well block); Bio-Rad CFX384™
F	96-well	Roche® LightCycler® 480 (96-well block)
G	384-well	Roche LightCycler 480 (384-well block)
R	100-well Rotor-Disc	QIAGEN Rotor-Gene Q

Table 2. Real-time PCR cyclers suitable for use with qBiomarker Copy Number PCR Assays

Type of instrument	Real-time PCR cyclers
Instruments that use ROX as a passive reference dye	All Applied Biosystems, TaKaRa, and Stratagene real-time PCR cyclers
Instruments that use fluorescein as a passive reference dye	Bio-Rad iCycler, iQ5, MyiQ, MyiQ2
Instruments that do not require a reference dye	Bio-Rad/MJ Research DNA Engine Opticon, DNA Engine Opticon 2, and Chromo4; Roche LightCycler 480; Eppendorf Mastercycler ep *realplex* 2, 2S, 4, 4S; QIAGEN Rotor-Gene Q; other instruments that do not require a reference dye

Important Notes

General precautions

We recommend the following guidelines.

- Use sterile pipet tips with filters.
- Store and extract positive materials (specimens, positive controls, and amplicons) separately from all other reagents, and add them to the reaction mix in a spatially separated facility.
- Physically separate the workspaces used for PCR setup and post-PCR processing operations. Decontaminate your PCR workspace and labware (pipets, tube racks, etc.) with UV light before each new use to render any contaminated DNA ineffective in PCR through the formation of thymidine dimers or with 10% bleach to chemically inactivate and degrade any DNA.
- Do not remove the PCR array plate from its protective sealed bag until immediately before use.
- Thaw all components thoroughly at room temperature (15–25°C) before starting an assay.
- When thawed, mix the components (by pipetting repeatedly up and down or by pulse vortexing) and centrifuge briefly.

DNA purification

The kits from QIAGEN shown in Table 3 are recommended for genomic DNA purification from the indicated sample types for use with qBiomarker Copy Number PCR Arrays or Assays.

Table 3. DNA purification kits recommended for use with qBiomarker Copy Number PCR Assays and Arrays

Sample material	Nucleic acid isolation kit	Catalog number (QIAGEN)
Fresh or frozen tissues or cultured cells	QIAamp DNA Mini Kit	51304 or 51306
	DNeasy Blood & Tissue Kit	69504 or 69506
Formalin-fixed, paraffin-embedded (FFPE) tissues	QIAamp DNA FFPE Tissue Kit	56404

Carry out the DNA purification according to the instructions in the kit handbooks, but with the following changes to the kit protocols.

- Always perform the optional RNase A digest to remove RNA. RNA contamination will cause inaccuracies in DNA concentration measurements.
- Elute with nuclease-free water or the elution buffer provided in the DNA purification kit. Do not use water or buffers treated with diethylpyrocarbonate (DEPC).

DNA quality control

All DNA samples should show consistent quality either by UV spectrophotometric measurements or by performance in real-time PCR using the DNA QC Plate.

Measuring DNA concentration and purity by UV spectrophotometry

Dilute samples and measure absorbance in 10 mM Tris·Cl, pH 8.0. An absorbance reading of 1.0 at 260 nm in a 1 cm detection path corresponds to a DNA concentration of 50 µg/ml. All DNA samples should meet the following criteria.

- Concentration, as measured by A_{260}, should be greater than 10 µg/ml.
- A_{260}/A_{280} ratio should be greater than 1.8.
- A_{260}/A_{230} ratio should be greater than 1.7.

Measuring DNA quality with the DNA QC Plate

DNA quality and consistency can be checked more reliably with the DNA QC Plate by real-time PCR measuring 7 reference genes. For a detailed procedure, see "Appendix A: Quality Control of Genomic DNA Using the DNA QC Plate", page 43.

qBiomarker Copy Number PCR Array plate layouts

Each qBiomarker Copy Number PCR Array shipment includes the array plates/discs and either 12 Optical Thin-Wall 8-Cap Strips (plate formats A and D), one Optical Adhesive Film (plate formats C, E, F, and G), or one Rotor-Disc Heat-Sealing Film (disc format R) per plate/disc.

Each 384-well array (formats E and G) also includes one set of four 384EZLoad Covers (cat. no. 338125) for each plate. Each 384EZLoad Cover is for single use only.

Plate layouts

The 96-well plate layout for a 23-gene panel (one plate for one sample) has 4 replicates for 23 genes and a multi-copy reference assay (MRef), arranged as shown in Figure 1.

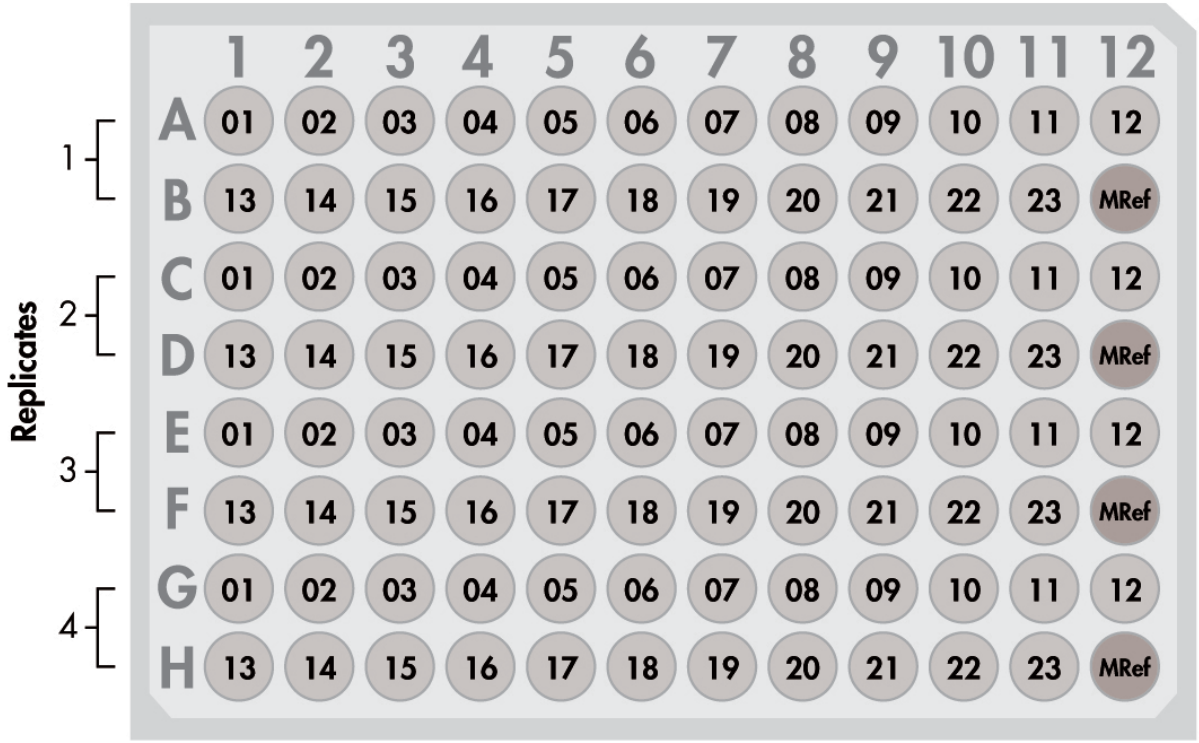


Figure 1. Plate layout for a 23-gene panel on a 96-well plate. The numbers in the cells each represent a single well for gene 1, gene 2, etc.

The 384-well plate layout for a 23-gene panel (one plate for 4 samples) has 16 replicates for 23 genes and a multi-copy reference assay (MRef), arranged as shown in Figure 2. A set of 4 rows contains 4 replicates for 23 genes.

The 384-well plate layout for a 95-gene panel (one plate for one sample) has 4 replicates of 95 genes and a multi-copy reference assay (MRef), arranged as shown in Figure 3.

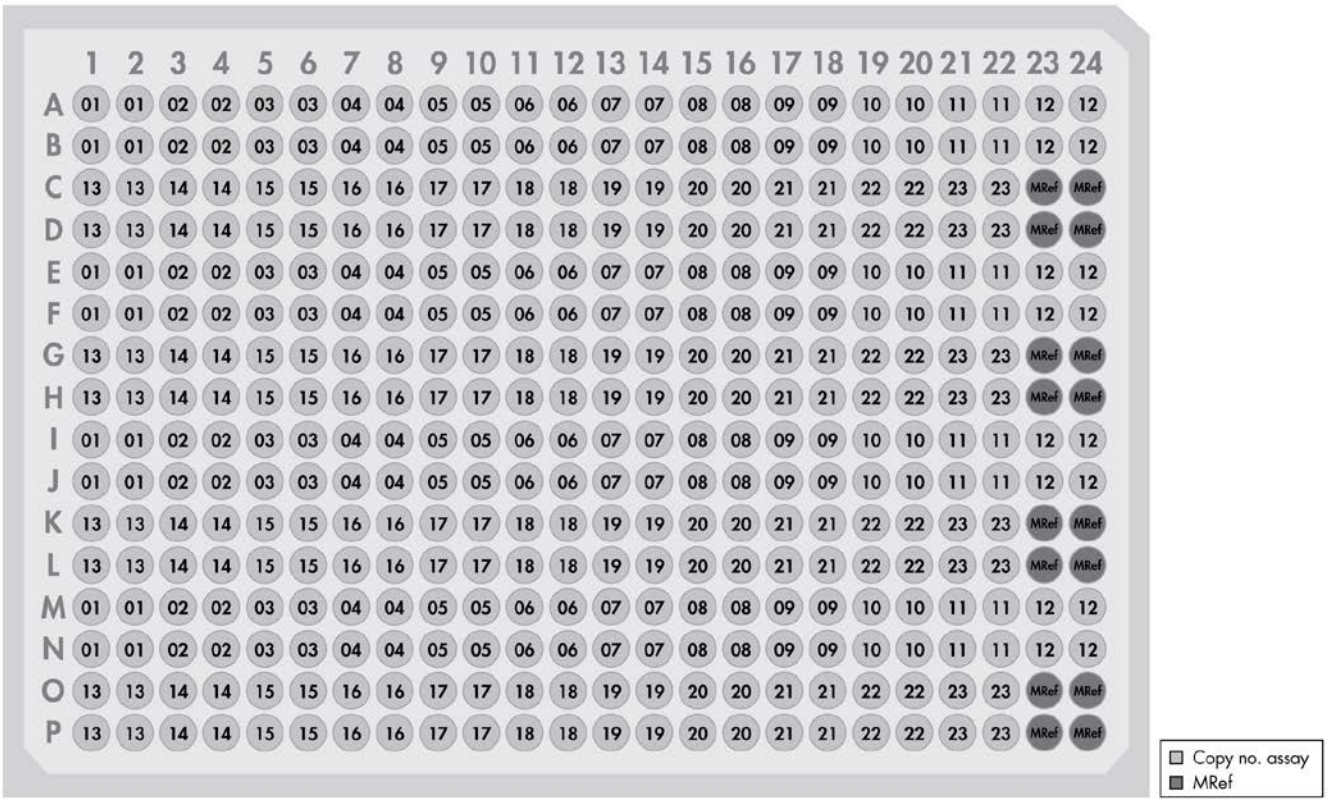


Figure 2. Plate layout for a 23-gene panel on a 384-well plate. The numbers in the cells each represent a single well for gene 1, gene 2, etc. The first set of 4 rows is repeated 3 more times so that one 384-well PCR array plate can be used to assay 4 samples.

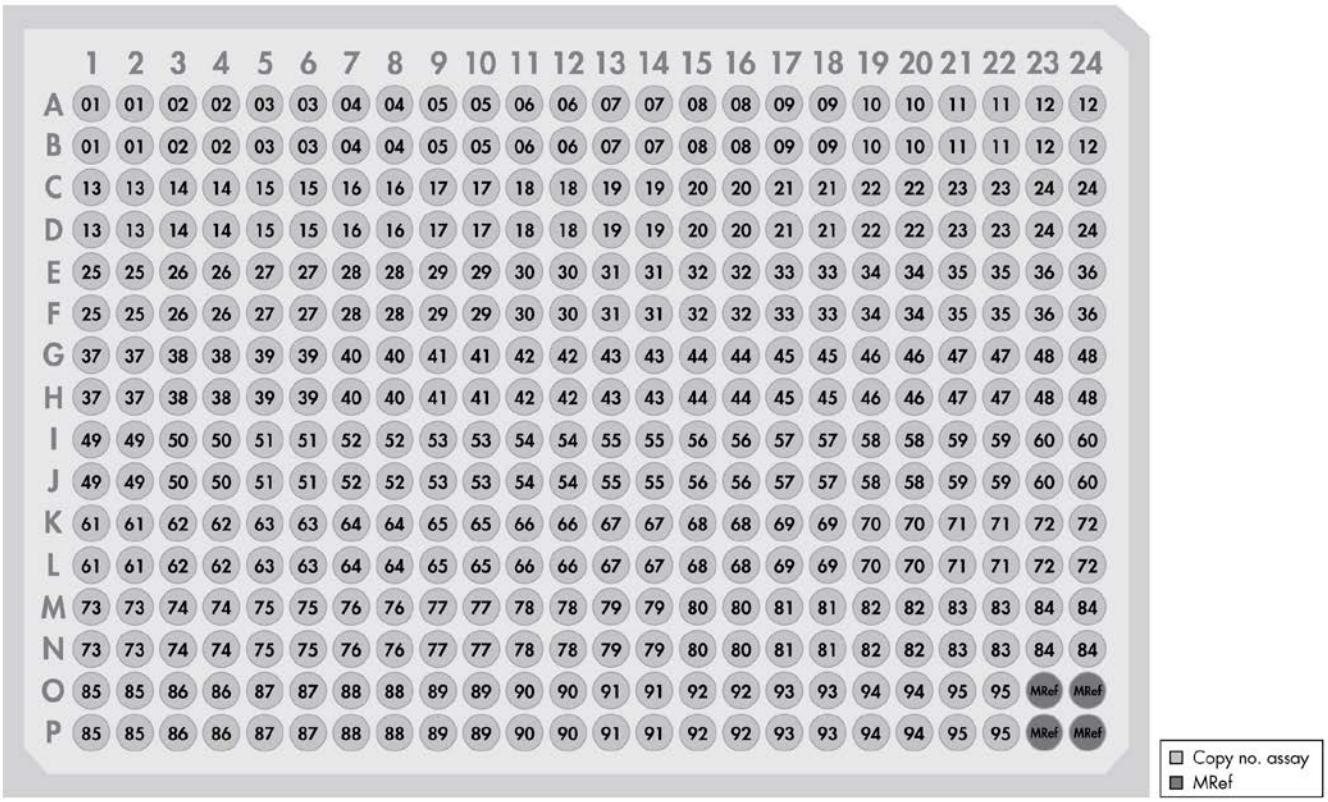


Figure 3. Plate layout for a 95-gene panel on a 384-well plate. The numbers in the cells each represent a single well for gene 1, gene 2, etc. One 384-well PCR array plate can be used to assay one sample.

The 100-well Rotor-Disc layout for a 23-gene panel (one disc for one sample) has 4 replicates of 23 genes and a multi-copy reference assay (MRef), arranged as shown in Figure 4.

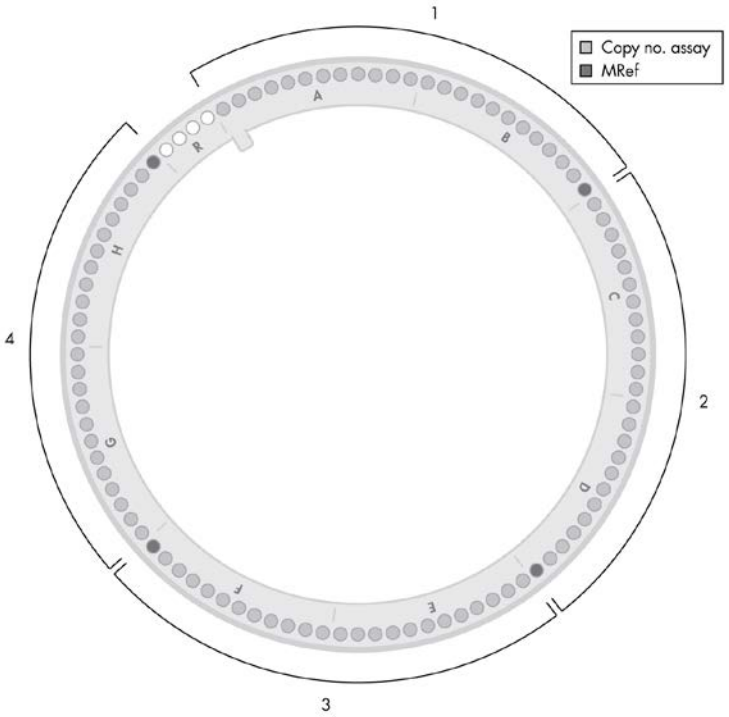


Figure 4. Disc layout for a 23-gene panel on a 100-well Rotor-Disc. One Rotor-Disc can be used to assay one sample.

Protocol: PCR Using qBiomarker Copy Number PCR Array Formats A, C, D, E, F, and G

This protocol is for use with qBiomarker Copy Number PCR Arrays in 96-well and 384-well plates. For use of qBiomarker Copy Number PCR Arrays in Rotor-Discs, see "PCR Using qBiomarker Copy Number PCR Arrays Format R", page 30. For use of individual qBiomarker Copy Number PCR Assays, see "PCR Using qBiomarker Copy Number PCR Assays", page 34.

Important points before starting

- Before beginning the procedure, read "Important Notes", pages 16–21.
- Make sure that the sample cell population contains at least 25% tumor cells for CNA profiling analysis.
- A calibrator sample is a DNA sample with known copy number for the targets of interest. When a calibrator sample or samples are used, we recommend that, if possible, the calibrator sample is from the same tissue type as the test sample. We also recommend that the DNA quality of the calibrator sample should be comparable to that of the test sample (see "Appendix A: Quality Control of Genomic DNA Using the DNA QC Plate", page 43, for details of assessment of sample quality).
  For fresh frozen test samples, DNA from a fresh frozen sample should also be used as a calibrator sample. For FFPE test samples, there are 2 options for a calibrator sample. DNA from a fresh frozen sample can be used as a calibrator sample, although the final copy number call may be largely influenced by the quality difference between the test sample and the calibrator sample. Alternatively, DNA from an FFPE sample can be used as a calibrator sample. In this case, it is preferable to include multiple calibrator samples in the study.
- Take time to familiarize yourself with the real-time PCR cycler before starting the protocol. Read the cycler user manual, and follow the manufacturer's instructions for proper cycler operation and maintenance.
- The chemically modified hot-start enzyme, along with other proprietary chemical components in the qBiomarker SYBR Mastermix, is essential for successful function of the arrays. Do not replace any of these reagents with other commercial counterparts.
- If a calibrator DNA sample is included, it should be run in the same real-time PCR cycler as the test samples.
- PCR array plates should only be used in the compatible real-time PCR cyclers listed in Table 1 (page 14). The PCR array plates will not fit properly into incompatible real-time PCR cyclers and may cause damage to the cycler.

- Pipetting accuracy and precision affects the consistency of results. Be sure that all pipets and instruments have been checked and calibrated according to the manufacturer's recommendations.
- Make sure that no bubbles are introduced into the wells of the PCR array during pipetting.
- Do not use DEPC-treated water. Use high-quality, nuclease-free water.
- If precipitates are present in the mastermix tube, warm the reagents at 42°C for 1 min and vortex briefly to dissolve. Repeat if necessary.

Procedure

1. **Briefly centrifuge the qBiomarker SYBR Mastermix (10–15 s) to bring the contents to the bottom of the tube.**

   Note: As the qBiomarker SYBR Mastermix contains HotStart DNA *Taq* Polymerase that is active only after heat activation, reactions can be prepared at room temperature (15–25°C).

2. **Prepare a reaction mix according to Table 4 for each sample.**

   Recommended amounts of genomic DNA per sample are shown in Table 5.

Table 4. Preparation of reaction mix

Array format:	96-well, 23-gene panel (Formats A, C, D, F)	384-well, 23-gene panel (Formats E, G)	384-well, 95-gene panel (Formats E, G)
qBiomarker SYBR Mastermix, 2x	1350 µl	550 µl	2100 µl
Genomic DNA	0.4–1.0 µg (fresh); 0.8–1.2 µg (FFPE)	0.2–0.5 µg (fresh); 0.4–1.0 µg (FFPE)	0.8–2.0 µg (fresh); 1.6–4.0 µg (FFPE)
Nuclease-free water	Variable	Variable	Variable
Total volume	**2700 µl***	**1100 µl†**	**4200 µl‡**

* This volume provides an excess of 300 µl per sample to allow for some imprecision in pipetting. † This volume provides an excess of 140 µl per sample to allow for some imprecision in pipetting. ‡ This volume provides an excess of 360 µl per sample to allow for some imprecision in pipetting.

Table 5. Recommended amounts of genomic DNA per sample

Sample type	96-well, 23-gene panel (Figure 1)	384-well, 23-gene panel (Figure 2)	384-well, 95-gene panel (Figure 3)
Fresh tissue	0.4–1.0 µg	0.2–0.5 µg	0.8–2.0 µg
FFPE	0.8–1.2 µg	0.4–1.0 µg	1.6–4.0 µg

3. **Carefully remove the PCR array plate from its sealed bag.**
4. **Recommended: Dispense the reaction mix into a RT² PCR Array Loading Reservoir to assist in loading.**
5. **Add reaction mix to each well of the PCR array plate as follows.**

   Note: Perform pipetting steps as precisely as possible to ensure that each well receives the required volume.

   Note: Change pipet tips following each addition to avoid any cross-contamination between the wells.

   - **96-well, 23-gene panel (Formats A, C, D, F):**

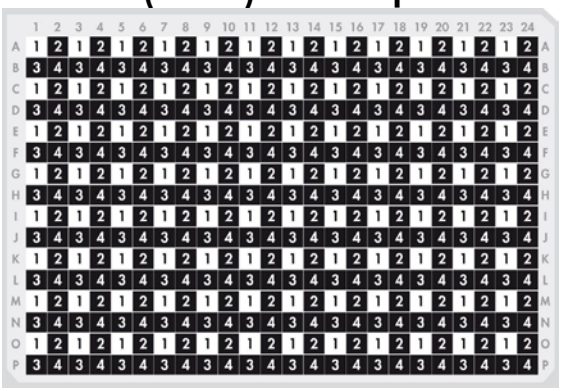
     Add 25 µl reaction mix to each well of the PCR array plate using a multichannel pipet with 8 tips.

   - **384-well, 23-gene panel (Formats E, G):**

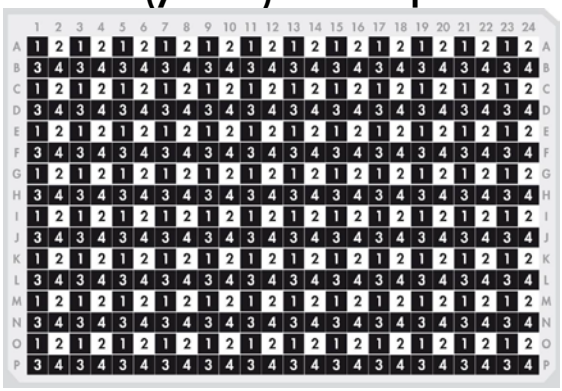
     Add 10 µl reaction mix to each well of the PCR array plate using a multichannel pipet with 8 tips.

     Each 384-well plate (23-gene panel) characterizes 4 samples in separate sets of 96-wells staggered from one another by only one well. The spacing between the tips of standard multichannel pipets should allow correct loading of samples into every other row and column. Be sure to load each sample into the correct set of wells using the 384EZLoad Covers provided and see Figure 5 as a guide.

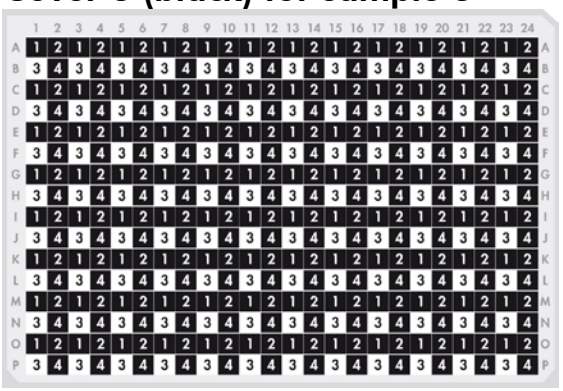
Cover 1 (white) for sample 1



Cover 2 (yellow) for sample 2



Cover 3 (black) for sample 3



Cover 4 (red) for sample 4

Figure 5. Loading 384-well, 23-gene panel, formats E, G. 384EZLoad Cover 1 (white): Place 384EZload Cover 1 (white) on the plate. Add 10 µl sample 1 reaction mix to the open wells (odd-numbered wells of rows A, C, E, G, I, K, M, and O). Remove and discard the cover. **384EZLoad Cover 2 (yellow):** Place 384EZload Cover 2 (yellow) on the plate. Add 10 µl sample 2 reaction mix to the open wells (even-numbered wells of rows A, C, E, G, I, K, M, and O). Remove and discard the cover. **384EZLoad Cover 3 (black):** Place 384EZload Cover 3 (black) on the plate. Add 10 µl sample 3 reaction mix to the open wells (odd-numbered wells of rows B, D, F, H, J, L, N, and P). Remove and discard the cover. **384EZLoad Cover 4 (red):** Place 384EZload Cover 4 (red) on the plate. Add 10 µl sample 4 reaction mix to the open wells (even-numbered wells of rows B, D, F, H, J, L, N, and P). Remove and discard the cover.

- **384-well, 95-gene panel (Formats E, G):**

  Add 10 µl reaction mix to each well of the PCR array plate using a multichannel pipet with 8 tips.

  Each 384-well plate (95-gene panel) characterizes one sample in 4 sets of 96-wells staggered from one another by only one well. The spacing between the tips of standard multichannel pipets should allow you to properly load the sample into every other row and column.

6. **Carefully but tightly seal the PCR array plate with the Optical Thin-Wall 8-Cap Strips (plate formats A and D) or with the Optical Adhesive Film (plate formats C, E, F, and G).**

7. **Remove any air bubbles in the wells of the PCR array plate by centrifuging the plate at 1000 rpm for 1 min (96-well plate, formats A, C, D, F) or 2000 rpm for 2 min (384-well plate, formats E, G).**
8. **Place the PCR array plate on ice while setting up the PCR cycling program in step 9.**

   Note: PCR array plates containing reaction mix that will not be processed immediately may be stored wrapped in aluminum foil at –20°C for up to one week until ready to run.
9. **Program the real-time cycler according to Table 6, 7, or 8, depending on the real-time cycler used. If prompted by your cycler software, select "Absolute Quantitation" to begin.**

   Note: For additional help with instrument setup, see the instrument-specific setup instructions and protocol files at www.SABiosciences.com/pcrarrayprotocolfiles.php.

Table 6. Cycling conditions* for Applied Biosystems, Bio-Rad,† Stratagene, and Eppendorf‡ cyclers

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
40	15 s	95°C	
	1 min	60°C	Perform fluorescence data collection.

* Recommended for the following cyclers: Applied Biosystems models 5700, 7000, 7300, 7500, 7700, 7900HT, StepOnePlus, ViiA 7; Bio-Rad models iCycler, iQ5, MyiQ, MyiQ2, CFX96, CFX384; Stratagene models Mx3000P, Mx3005P, Mx4000P; Eppendorf Mastercycler ep *realplex* models 2, 2S, 4, 4S.

† For Bio-Rad models CFX96 and CFX384: adjust the ramp rate to 1°C/s.

‡ For Eppendorf Mastercyler ep *realplex* models 2, 2S, 4, and 4S: for the Silver Thermoblock, adjust the ramp rate to 26%; for the Aluminum Thermoblock, adjust the ramp rate to 35%. Refer to the Instrument Setup Guide at www.SABiosciences.com/pcrarrayprotocolfiles.php for detailed setup instructions.

Table 7. Cycling conditions for Roche LightCycler 480*

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
45	15 s	95°C	
	1 min	60°C	Perform fluorescence data collection.

* Recommended for the Roche LightCycler 480. If using a Roche LightCycler 480, adjust the ramp rate to 1°C/s. Refer to the "Instrument Setup Guide" at www.SABiosciences.com/pcrarrayprotocolfiles.php for more information on other required changes to settings for Melt Curve Acquisition.

Table 8. Cycling conditions for Bio-Rad and Takara cyclers and other cyclers†

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
40	15 s	95°C	
	30–40 s	55°C	Perform fluorescence data collection. Different cyclers need different lengths of time to detect the fluorescent signal. Choose the appropriate time for the annealing step (55°C) for your cycler.
	30 s	72°C	

† Recommended for the following cyclers: Bio-Rad/MJ Research models Chromo4, DNA Engine Opticon, DNA Engine Opticon 2; Takara TP-800; all other cyclers excluding Rotor-Gene cyclers.

10. Place one PCR array plate in the real-time PCR cycler. Use a compression pad with the optical film-sealed plate formats (plate formats C, E, F, and G) if recommended by the manufacturer. Start the run.

11. **After the run is finished, calculate the threshold cycle (C_T) for each well using the real-time cycler software, as described in the following steps.**

    Note: If using the Roche LightCycler 480, there are 2 options for data analysis: using the second derivate max setting (in this case, there is no need to calculate the C_T) or using "Fit Points" (in this case, the C_T should be defined manually as described in step 13).

12. **Define the baseline by choosing the automated baseline option if the cycler has the adaptive baseline function. If the cycler does not have the adaptive baseline function, set the baseline manually. To set the baseline manually, use the linear view of the amplification plots to determine the earliest visible amplification. Set the cycler to use the readings from cycle number 2 up to 2 cycles before the earliest visible amplification.**

13. **Manually define the threshold by using the log view of the amplification plots. Choose a threshold value above the background signal but within the lower one-third to lower half of the linear phase of the amplification plot.**

    Note: Ensure that the threshold values are the same across all qBiomarker Copy Number PCR Array runs in the same analysis. The absolute position of the threshold is less critical than its consistent position across arrays.

14. **Export the C_T values for all wells to a blank Excel® spreadsheet for use with the qBiomarker Copy Number PCR Array Data Analysis Web-based software.**

    Note: qBiomarker Copy Number PCR Array Data Analysis Software is available at http://pcrdataanalysis.sabiosciences.com/cnv.

15. **Recommended: Perform dissociation (melting) curve analysis to verify PCR specificity. Run a melting curve program and generate a first derivative dissociation curve for each well using the real-time cycler software. A single peak should appear in each reaction.**

    Note: If your cycler does not have a default melting curve program, run the following program instead: 95°C, 1 min; 65°C, 2 min (optics off); 65°C to 95°C at 2°C/min (optics on).

    Note: For cycler-specific melting curve analysis settings, please refer to the Instrument Setup Guide for your cycler at www.SABiosciences.com/pcrarrayprotocolfiles.php.

    Note: Plates can be stored at –20°C wrapped in aluminum foil and melting curve analysis performed at a later time. When ready to perform melting curve analysis, warm the plate to room temperature (15–25°C), place it in the real-time cycler, and run the melting curve analysis program.

Note: Visually inspect the plate after the run for any signs of evaporation from any of the wells. If evaporation is observed, note which wells are affected, as this may affect the results of data analysis.

Note: Do not open any previously processed qBiomarker Copy Number PCR Array. Removing the Optical Thin-Wall 8-Cap Strips or the Optical Adhesive Film from a qBiomarker Copy Number PCR Array releases PCR product into the air where it may contaminate and affect the results of future real-time PCR experiments.

Protocol: PCR Using qBiomarker Copy Number PCR Array Format R

This protocol is for use with qBiomarker Copy Number PCR Arrays in 100-well Rotor-Discs. For use of qBiomarker Copy Number PCR Arrays in 96-well and 384-well plates, see “PCR Using qBiomarker Copy Number PCR Arrays Formats A, C, D, E, F, and G”, page 22. For use of individual qBiomarker Copy Number PCR Assays, see “PCR Using qBiomarker Copy Number PCR Assays”, page 34.

Important points before starting

- Before beginning the procedure, read “Important Notes”, pages 16–21.
- Make sure that the sample cell population contains at least 25% tumor cells for CNA profiling analysis.
- A calibrator sample is a DNA sample with known copy number for the targets of interest. When a calibrator sample or samples are used, we recommend that, if possible, the calibrator sample is from the same tissue type as the test sample. We also recommend that the DNA quality of the calibrator sample is comparable to that of the test sample (see "Appendix A: Quality Control of Genomic DNA Using the DNA QC Plate", page 43, for details of assessment of sample quality).
  For fresh frozen test samples, DNA from a fresh frozen sample should also be used as a calibrator sample. For FFPE test samples, there are 2 options for a calibrator sample. DNA from a fresh frozen sample can be used as a calibrator sample, although the final copy number call may be largely influenced by the quality difference between the test sample and the calibrator sample. Alternatively, DNA from an FFPE sample can be used as a calibrator sample. In this case, it is preferable to include multiple calibrator samples in the study.
- Only qBiomarker Copy Number PCR Array Format R and qBiomarker SYBR ROX FAST Mastermix should be used with Rotor-Gene cyclers.
- Take time to familiarize yourself with the real-time PCR cycler before starting the protocol. Read the cycler user manual, and follow the manufacturer’s instructions for proper cycler operation and maintenance.
- The chemically modified hot-start enzyme, along with other proprietary chemical components in the qBiomarker SYBR Mastermix, is essential for successful function of the arrays. Do not replace any of these reagents with other commercial counterparts.
- If a calibrator DNA sample is included, it should be run in the same real-time PCR cycler as the test samples.

- Pipetting accuracy and precision affects the consistency of results. Be sure that all pipets and instruments have been checked and calibrated according to the manufacturer's recommendations.
- Make sure that no bubbles are introduced into the wells of the PCR array during pipetting.
- Do not use DEPC-treated water. Use high-quality, nuclease-free water.
- If precipitates are present in the mastermix tube, warm the reagents at 42°C for 1 min and vortex briefly to dissolve. Repeat if necessary.

Procedure

1. **Briefly centrifuge the qBiomarker SYBR Mastermix (10–15 s) to bring the contents to the bottom of the tube.**

   Note: As the qBiomarker SYBR Mastermix contains HotStart DNA *Taq* Polymerase that is active only after heat activation, reactions can be prepared at room temperature (15–25°C).

2. **Prepare a reaction mix according to Table 9 for each sample.**

   For genomic DNA from fresh tissue samples, it is recommended to start with 0.4–1.0 µg DNA for each sample. Due to the fragmented nature of DNA extracted from FFPE samples, it is recommended to use 0.8–1.2 µg DNA for each sample.

Table 9. Preparation of reaction mix

Array format:	Rotor-Disc 100
qBiomarker SYBR Mastermix, 2x	1150 µl
Genomic DNA	0.4–1.0 µg (fresh) or 0.8–1.2 µg (FFPE)
Nuclease-free water	Variable
Total volume	**2300 µl***

* This volume provides an excess of 300 µl per sample to allow for some imprecision in pipetting.

3. **Carefully remove the PCR array Rotor-Disc from its sealed bag. Slide the Rotor-Disc into the Rotor-Disc 100 Loading Block using the tab at position A1 and the tube guide holes.**
4. **Add 20 µl reaction mix to each well of the Rotor-Disc. Proceed to step 5.**

Note: Change pipet tips following each pipetting step to avoid cross-contamination between the wells.

Note: Reaction mix can be dispensed manually or using the QIAgility® (www.qiagen.com/goto/QIAgility).

Note: Although wells 97–100 do not contain assays, it is essential to add reaction mix for optimized balancing of the Rotor-Disc.

5. **Carefully seal the Rotor-Disc with Rotor-Disc Heat-Sealing Film using the Rotor-Disc Heat Sealer.**

   For detailed instructions, see the *Rotor-Gene Q User Manual*.

   Note: The Rotor-Disc containing PCR components mix may be stored at –20°C wrapped in aluminum foil for up to one week if desired.

6. **Program the real-time cycler according to Table 10.**

   Note: For additional help with instrument setup, see the instrument-specific setup instructions and protocol files at www.SABiosciences.com/pcrarrayprotocolfiles.php.

Table 10. Cycling conditions for Rotor-Gene cyclers

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
40	15 s	95°C	
	30 s	60°C	Perform fluorescence data collection.

7. **Insert the Rotor-Disc into the Rotor-Disc 100 Rotor and secure with the Rotor-Disc 100 Locking Ring. Start the run.**

   For detailed instructions, see the *Rotor-Gene Q User Manual*.

8. **After the run is finished, calculate the threshold cycle (C_T) for each well using the real-time cycler software. To define the baseline, select "Dynamic Tube" (default analysis setting) to ensure the average background of each well is determined just before amplification commences.**

   Optional: Select "Ignore First". Fluorescent signal from the initial cycles may not be representative of the remainder of the run. Thus, better results may be achieved if the initial cycles are ignored. Up to 5 cycles can be ignored.

   Optional: Select "Noise Slope Correction". Selection of this option can improve data whose baseline (initial cycles) is noticeably sloped. "Noise

Slope Correction" improves the data when raw data backgrounds are observed to slope upward or downward before the takeoff point (C_T).

Note: Ensure that the settings are the same across all qBiomarker Copy Number PCR Array runs in the same analysis.

9. **Manually define the threshold by using the log view of the amplification plots. Choose a threshold value above the background signal but within the lower one-third to lower half of the linear phase of the amplification plot.**

   Note: Ensure that the threshold values are the same across all qBiomarker Copy Number PCR Array runs in the same analysis. The absolute position of the threshold is less critical than its consistent position across arrays.

10. **Export the C_T values for all wells to a blank Excel spreadsheet for use with the qBiomarker Copy Number PCR Array Data Analysis Web-based software.**

    Note: qBiomarker Copy Number PCR Array Data Analysis Software is available at http://pcrdataanalysis.sabiosciences.com/cnv.

11. **Recommended: Perform dissociation (melting) curve analysis to verify PCR specificity. Run a melting curve program and generate a first derivative dissociation curve for each well using the real-time cycler software. A single peak should appear in each reaction.**

    Note: Melting curve analysis can be added during creation of the Rotor-Gene Q PCR program.

    Note: For Rotor-Gene Q melting curve analysis settings, refer to the Instrument Setup Guide at www.SABiosciences.com/pcrarrayprotocolfiles.php.

    Note: Rotor-Discs can be stored at –20°C wrapped in aluminum foil and melting curve analysis performed at a later time. When ready to perform melting curve analysis, warm the plate to room temperature (15–25°C), place it in the real-time cycler, and run the melting curve analysis program.

    Note: Visually inspect the Rotor-Disc after the run for any signs of evaporation from any of the wells. If evaporation is observed, note which wells are affected, as this may affect the results of data analysis.

    Note: Do not open any previously processed qBiomarker Copy Number PCR Array. Removing the film from qBiomarker Copy Number PCR Arrays releases PCR product into the air where it may contaminate and affect the results of future real-time PCR experiments.

Protocol: PCR Using qBiomarker Copy Number PCR Assays

This protocol is for use with qBiomarker Copy Number PCR Assays. For use of qBiomarker Copy Number PCR Arrays in 96-well and 384-well plates, see “PCR Using qBiomarker Copy Number PCR Array Formats A, C, D, E, F, G”, page 22. For use of qBiomarker Copy Number PCR Arrays in Rotor-Discs, see “PCR Using qBiomarker Copy Number PCR Arrays Format R”, page 30.

Important points before starting

- Before beginning the procedure, read “Important Notes”, pages 16–21.
- Make sure that the sample cell population contains at least 25% tumor cells for CNA profiling analysis.
- A calibrator sample is a DNA sample with known copy number for the targets of interest. When a calibrator sample or samples are used, we recommend that, if possible, the calibrator sample is from the same tissue type as the test sample. We also recommend that the DNA quality of the calibrator sample is comparable to that of the test sample (see "Appendix A: Quality Control of Genomic DNA Using the DNA QC Plate", page 43, for details of assessment of sample quality).
  For fresh frozen test samples, DNA from a fresh frozen sample should also be used as a calibrator sample. For FFPE test samples, there are 2 options for a calibrator sample. DNA from a fresh frozen sample can be used as a calibrator sample, although the final copy number call may be largely influenced by the quality difference between the test sample and the calibrator sample. Alternatively, DNA from an FFPE sample can be used as a calibrator sample. In this case, it is preferable to include multiple calibrator samples in the study.
- Take time to familiarize yourself with the real-time PCR cycler before starting the protocol. See the cycler user manual, and follow the manufacturer’s instructions for proper cycler operation and maintenance.
- The chemically modified hot-start enzyme, along with other proprietary chemical components in the qBiomarker Copy Number Reference Mix and qBiomarker SYBR Mastermix, is essential for successful function of the assays. Do not replace any of these reagents with other commercial counterparts.
- If a calibrator DNA sample is included, it should be run in the same real-time PCR cycler as the test samples.
- Pipetting accuracy and precision affects the consistency of results. Be sure that all pipets and instruments have been checked and calibrated according to the manufacturer’s recommendations.

- Make sure that no bubbles are introduced into the wells of the PCR plate during pipetting.
- Do not use DEPC-treated water. Use high-quality, nuclease-free water.
- If precipitates are present in the mastermix tube, warm the reagents at 42°C for 1 min and vortex briefly to dissolve. Repeat if necessary.

Procedure

1. **Briefly centrifuge the qBiomarker SYBR Mastermix (10–15 s) to bring the contents to the bottom of the tube.**

   Note: As the qBiomarker SYBR Mastermix contains HotStart DNA *Taq* Polymerase that is active only after heat activation, reactions can be prepared at room temperature (15–25°C).

2. **Prepare a reaction mix according to Table 11.**

   For genomic DNA from fresh or frozen tissue samples, it is recommended to start with 4 ng DNA per well if setting up reactions in 96-well plates or 100-well Rotor-Discs or 2 ng DNA per well if setting up reactions in 384-well plates. Due to the fragmented nature of DNA extracted from FFPE samples, it is recommended to use 8–20 ng DNA per well if setting up reactions in 96-well plates or 100-well Rotor-Discs or 4–10 ng DNA per well if setting up reactions in 384-well plates.

   Note: It is recommended to prepare a reaction mix for a minimum of 4 replicate reactions for each sample. Prepare 5% more mix than is required to allow for imprecision in pipetting.

Table 11. Preparation of reaction mix

Format:	96-well plate	Rotor-Disc	384-well plate
Number of reactions:	**4**	**4**	**4**
qBiomarker SYBR Mastermix, 2x	50 µl	40 µl	20 µl
Genomic DNA	16 ng (fresh); 32–80 ng (FFPE)	16 ng (fresh); 32–80 ng (FFPE)	8 ng (fresh); 16–40 ng (FFPE)
qBiomarker Copy Number PCR Assay	5 µl	4 µl	2 µl
Nuclease-free water	Variable	Variable	Variable
Total volume	**100 µl**	**80 µl**	**40 µl**

3. **Add 25 µl reaction mix to each well of a 96-well plate or 20 µl reaction mix to each well of a Rotor-Disc 100 or 10 µl reaction mix to each well of a 384-well plate using a single-channel pipet.**

   Note: Change pipet tips following each addition to avoid any cross-contamination between the wells.

4. **Tightly seal the PCR plate with Optical Thin-Wall 8-Cap Strips or Optical Adhesive Film. If using a Rotor-Disc or Strip Tubes, tightly seal using the Rotor-Disc Heat Sealing Film or caps.**
5. **Remove any air bubbles in any of the plate wells by centrifuging the plate at 1000 rpm for 1 min (96-well plate, formats A, C, D, F), 2000 rpm for 2 min (384-well plate, formats E, G).**

   This step is not necessary for Rotor-Disc, format R.

6. **Place the PCR plate on ice while setting up the PCR cycling program in step 7.**
7. **Program the real-time cycler according to Table 12, 13, 14, or 15, depending on the real-time cycler used. If prompted by your cycler software, select "Absolute Quantitation" to begin.**

   Note: For additional help with instrument setup, see the instrument-specific setup instructions and protocol files at www.SABiosciences.com/pcrarrayprotocolfiles.php.

Table 12. Cycling conditions* for Applied Biosystems, Bio-Rad,† Stratagene, and Eppendorf‡ cyclers

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
40	15 s	95°C	
	1 min	60°C	Perform fluorescence data collection.

* Recommended for the following cyclers: Applied Biosystems models 5700, 7000, 7300, 7500, 7700, 7900HT, StepOnePlus, ViiA 7; Bio-Rad models iCycler, iQ5, MyiQ, MyiQ2, CFX96, CFX384; Stratagene models Mx3000P, Mx3005P, Mx4000P; Eppendorf Mastercycler ep *realplex* models 2, 2S, 4, 4S.

† For Bio-Rad models CFX96 and CFX384: adjust the ramp rate to 1°C/s.

‡ For Eppendorf Mastercyler ep *realplex* models 2, 2S, 4, and 4S: for the Silver Thermoblock, adjust the ramp rate to 26%; for the Aluminum Thermoblock, adjust the ramp rate to 35%. Refer to the Instrument Setup Guide at www.SABiosciences.com/pcrarrayprotocolfiles.php for detailed setup instructions.

Table 13. Cycling conditions for Roche LightCycler 480¶

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
45	15 s	95°C	
	1 min	60°C	Perform fluorescence data collection.

¶ Recommended for the Roche LightCycler 480. If using a Roche LightCycler 480, adjust the ramp rate to 1°C/s. Refer to the "Instrument Setup Guide" at www.SABiosciences.com/pcrarrayprotocolfiles.php for more information on other required changes to settings for Melt Curve Acquisition.

Table 14. Cycling conditions for Rotor-Gene cyclers

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
40	15 s	95°C	
	30 s	60°C	Perform fluorescence data collection.

Table 15. Cycling conditions for Bio-Rad and Takara cyclers and other cyclers*

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
40	15 s	95°C	
	30–40 s	55°C	Perform fluorescence data collection. Different cyclers need different lengths of time to detect the fluorescent signal. Choose the appropriate time for the annealing step (55°C) for your cycler.
	30 s	72°C	

* Recommended for the following cyclers: Bio-Rad/MJ Research models Chromo4, DNA Engine Opticon, DNA Engine Opticon 2; Takara TP-800; all other cyclers.

8. **Place the PCR plate/disc in the real-time PCR cycler. Use a compression pad with the optical film-sealed plate formats (plate formats C, E, F, and G) if recommended by the manufacturer. Start the run.**
9. **After the run is finished, calculate the threshold cycle (C_T) for each well using the real-time cycler software.**

   For assays performed using a Rotor-Gene cycler, follow steps 8 and 9, page 32 and 33. For assays performed using all other cyclers, follow steps 11–13, page 28.
10. **Export the C_T values for all wells to a blank Excel spreadsheet for use with the qBiomarker Copy Number PCR Assay Data Analysis Web-based software.**

    Note: qBiomarker Copy Number PCR Assay Data Analysis Software is available at http://pcrdataanalysis.sabiosciences.com/cnv.
11. **Recommended: Perform dissociation (melting) curve analysis to verify PCR specificity. Run a melting curve program and generate a first derivative dissociation curve for each well using the real-time cycler software. A single peak should appear in each reaction.**

    Note: If your cycler does not have a default melting curve program, run the following program instead: 95°C, 1 min; 65°C, 2 min (optics off); 65°C to 95°C at 2°C/min (optics on).

Note: For cycler-specific melting curve analysis settings, please refer to the Instrument Setup Guide for your cycler at www.SABiosciences.com/pcrarrayprotocolfiles.php.

Note: Plates/discs can be stored at –20°C wrapped in aluminum foil and melting curve analysis performed at a later time. When ready to perform melting curve analysis, warm the plate/disc to room temperature (15–25°C), place it in the real-time cycler, and run the melting curve analysis program.

Note: Visually inspect the plate/disc after the run for any signs of evaporation from any of the wells. If evaporation is observed, note which wells are affected, as this may affect the results of data analysis.

Note: Do not open any previously processed qBiomarker Copy Number PCR Assay plates/discs. Removing the strips or film releases PCR product into the air where it may contaminate and affect the results of future real-time PCR experiments.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
C_T values very high across all wells for a sample	
a) Cycling program incorrect	Make sure to program the real-time PCR cycler with the temperature profile shown in the protocols. Repeat the PCR with corrected settings if necessary.
b) Poor DNA quality	Check the DNA quality using the DNA QC Plate (see "Appendix A: Quality Control of Genomic DNA Using the DNA QC Plate", page 43) or on an agarose gel* to see if the DNA is degraded. If the DNA is not degraded, it could be of insufficient purity. We recommend using one of the kits indicated in Table 3, page 16, for isolation of high-quality DNA.
c) Too little DNA used	Make sure that the DNA has been properly quantified. During the DNA purification process, it is essential to perform an RNase digestion. RNA contamination in the DNA sample will lead to overestimation of DNA quantity. Note that larger amounts of DNA are recommended when working with FFPE samples.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Potentially amplified genes have lower copy number and potentially deleted genes have higher copy number

Non-recurrent events	Copy number loss of large chromosomal segments containing the potentially amplified genes can occur when the genes are unrelated to the tumor growth. Similarly, copy number gain can occur for tumor suppressor genes. Chromosomes or chromosome segments containing tumor suppressor genes that are inactivated by point mutations can also show copy number gain.

Copy number is lower than expected in heterogeneous samples

Non-tumor cells in the sample	In the presence of non-tumor cells that have normal diploid genomes, the copy number call is dependent on the copy number of the target gene in the cancer cells and the amount of non-tumor cells in the heterogeneous sample. If the percentage of non-tumor cells in the sample can be estimated, the copy number for a gene can be estimated based on Table 16.

Table 16. Evaluating gene copy number for cancer cells in the presence of non-tumor cells

Gene copy number in cancer cells	Expected copy number in sample with indicated percentage of normal cells				
	12.5%	**25%**	**50%**	**75%**	**87.5%**
0	0.25	0.5	1	1.5	1.75
3	2.9	2.8	2.5	2.3	2.1
4	3.8	3.5	3.0	2.5	2.3
6	5.5	5.0	4.0	3.0	2.5
8	7.3	6.5	5.0	3.5	2.8
10	9.0	8.0	6.0	4.0	3.0
12	10.8	9.5	7.0	4.5	3.3
14	12.5	11.0	8.0	5.0	3.5
16	14.3	12.5	9.0	5.5	3.8
18	16.0	14.0	10.0	6.0	4.0
20	17.8	15.5	11.0	6.5	4.3
22	19.5	17.0	12.0	7.0	4.5
24	21.3	18.5	13.0	7.5	4.8
26	23.0	20.0	14.0	8.0	5.0
28	24.8	21.5	15.0	8.5	5.3
30	26.5	23.0	16.0	9.0	5.5
32	28.3	24.5	17.0	9.5	5.8

Appendix A: Quality Control of Genomic DNA Using the DNA QC Plate

Sample DNA quality can affect the performance of qBiomarker Copy Number PCR Arrays and Assays. For DNA extracted from FFPE samples, different degrees of cross-linking and fragmentation may result in suboptimal PCR results. When uncertain of the sample quality, it is recommended to check the DNA quality first using the DNA QC Plate.

The DNA QC Plate is designed to measure the C_T values of 7 reference genes. When the DNA is highly cross-linked or fragmented, the C_T values from these 7 genes will be much higher than those from the same amount of high-quality DNA. DNA QC Plates are available in formats A, C, D, and F (96-well plates; Figure 6), formats E, G (384-well plates, see Product Sheet or Web site for plate layout), or format R (Rotor-Disc 100, see Product Sheet or Web site for plate layout). Each 96-well format DNA QC Plate can be used for quality control of 12 DNA samples. Each 384-well format DNA QC Plate can be used for quality control of 48 DNA samples. Each Rotor-Disc 100 format DNA QC Plate can be used for quality control of 12 DNA samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BRAF	BRAF	BRAF	BRAF	BRAF	BRAF	BRAF	BRAF	BRAF	BRAF	BRAF	BRAF
B	KRAS	KRAS	KRAS	KRAS	KRAS	KRAS	KRAS	KRAS	KRAS	KRAS	KRAS	KRAS
C	HRAS	HRAS	HRAS	HRAS	HRAS	HRAS	HRAS	HRAS	HRAS	HRAS	HRAS	HRAS
D	NRAS	NRAS	NRAS	NRAS	NRAS	NRAS	NRAS	NRAS	NRAS	NRAS	NRAS	NRAS
E	MEK1	MEK1	MEK1	MEK1	MEK1	MEK1	MEK1	MEK1	MEK1	MEK1	MEK1	MEK1
F	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA
G	PTEN	PTEN	PTEN	PTEN	PTEN	PTEN	PTEN	PTEN	PTEN	PTEN	PTEN	PTEN
H	SMPC	SMPC	SMPC	SMPC	SMPC	SMPC	SMPC	SMPC	SMPC	SMPC	SMPC	SMPC

Figure 6. Plate layout for DNA QC Plate, 96-well plate. The reference genes are indicated for each well.

Important points before starting

- See "Important points before starting", page 22, before beginning the procedure.

Procedure

A1. Prepare a reaction mix according to Table 17.

Note: Use the qBiomarker Probe Mastermix included with the DNA QC Plate.

Note: It is recommended to prepare a reaction mix for 8.4 replicate PCRs for each sample. Prepare more mix than is required so as to allow for imprecision in pipetting.

Table 17. Preparation of reaction mix for 8.4 replicate PCRs

Number of reactions:	**96-well plate**	**384-well plate**	**Rotor-Disc 100**
qBiomarker Probe Mastermix, 2x (included with DNA QC Plate)	105 µl	42 µl	84 µl
Genomic DNA	40 ng	16 ng	32 ng
Nuclease-free water	Variable	Variable	Variable
Total volume	**210 µl**	**84 µl**	**168 µl**

A2. Carefully remove the DNA QC Plate from its sealed bag.

A3. Recommended: Dispense the reaction mix into a RT² PCR Array Loading Reservoir to assist in loading.

A4. Add reaction mix to each well of the DNA QC Plate as follows.

For 96-well plate: add 25 µl reaction mix.

For 384-well plate: add 10 µl reaction mix.

For Rotor-Disc 100: add 20 µl reaction mix.

Note: Change pipet tips following each addition to avoid any cross-contamination between the wells.

A5. Carefully but tightly seal the DNA QC Plate with Optical Thin-Wall 8-Cap Strips (formats A and D), Optical Adhesive Film (formats C, E, F, and G), or Rotor-Disc Heat-Sealing Film (format R).

A6. Remove any air bubbles in any of the plate wells by centrifuging the plate at 1000 rpm for 1 min (96-well plate, formats A, C, D, F), 2000 rpm for 2 min (384-well plate, formats E, G).

This step is not necessary for the Rotor-Disc, format R.

A7. Place the DNA QC Plate on ice while setting up the PCR cycling program in step A8.

A8. Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 18.

Table 18. Cycling conditions

Cycles	Duration	Temperature	Comments
1	10 min	95°C	Initial PCR activation step.
40	15 s	95°C	Denaturation step.
	60 s	60°C	Annealing and extension. Detect and record FAM™ fluorescence from every well during the annealing/extension step of each cycle.

A9. Place the DNA QC Plate in the real-time PCR cycler. Use a compression pad with the optical film-sealed plate formats (plate formats C, E, F, and G) if recommended by the manufacturer. For format R, insert the Rotor-Disc 100 into the Rotor-Disc 100 Rotor and secure with the Rotor-Disc 100 Locking Ring. Start the run.

A10. After the run is finished, calculate the threshold cycle (C_T) for each well using the real-time cycler software, as described in the following steps.

Note: Ensure the settings are the same across all PCR assay runs in the same analysis.

A11. For formats A, C, D, E, F, G: We highly recommend manually setting the baseline and threshold values (see Table 19 for examples). To define the baseline value, use the linear view of the amplification plots and set the instrument to use the readings from cycle 5 up to 2 cycles before the earliest visible amplification, usually around cycle 15, but not greater than cycle 20.

To define the threshold value, use the log view of the amplification plots and place the threshold value above the background signal but within the lower half to one-third of the linear phase of the amplification plot.

A12. For format R: To define the baseline value, select "Ignore First". Manually define the threshold value by using the log view of the amplification plots. Select a threshold value above the background signal. The threshold value should be in the lower half to one-third of the linear phase of the amplification plot. A threshold setting of 0.03 is recommended as a reference.

Fluorescent signal from the initial cycles may not be representative of the remainder of the run. Therefore, better results may be achieved if the initial cycles are ignored. Up to 5 cycles can be ignored.

Table 19 shows typical settings for selected real-time PCR cyclers. Actual settings may differ.

Table 19. Typical baseline and threshold settings

Instruments	Baseline	Threshold
Applied Biosystems 7900HT	5–18 cycles	0.1
Applied Biosystems 7500	5–18 cycles	0.1
Stratagene Mx3000P, Mx3005P	Varies	0.1

A13. Export the resulting C_T values for all wells to a blank Excel spreadsheet.

Data analysis

To determine the quality of DNA samples based on the C_T results, first ensure that the C_T of the SMPC assay for each sample is consistent at ~22 (formats A, C, D, E, F, G) or ~21 (format R). If not, adjust the baseline and threshold setting to achieve that value. Then calculate the average of the lowest 6 C_T values for each sample tested. (The highest C_T value is not included in calculating the average since some samples may contain a homozygous deletion for one of the 7 genes included on the DNA QC plate. This deleted gene will give a high C_T value.) The typical average C_T value for high-quality DNA from fresh tissue samples should be <29 (based on the baseline and threshold setup outlined above). Samples of lower quality (average C_T value >29) may not give optimal results.

For DNA extracted from FFPE samples, an average C_T value <32 for the lowest 6 C_T values (when using 5 ng genomic DNA in 25 µl reaction volume, 2 ng genomic DNA input in 10 µl reaction volume, or 4 ng genomic DNA in a 20 µl reaction volume) indicates sufficient quality for CNA profiling analysis. Samples of lower quality (average C_T value >32) may not yield optimal results or may

require more input DNA (estimate the amount fold increase needed to make the average C_T value <32).

Note: When a calibrator sample is used in copy number analysis, we recommend that the average C_T value of the calibrator sample (determined as described above) should be within 4 C_T values of that of a test sample.

Appendix B: Data Analysis Using the $\Delta\Delta C_T$ Method

At the qBiomarker Copy Number PCR Array and Assay Data Analysis Web portal (http://pcrdataanalysis.sabiosciences.com/cnv), C_T data can be entered and the Web-based software will automatically perform quantification using the $\Delta\Delta C_T$ method as described below. Results are presented in a tabular format.

Data analysis using the qBiomarker Copy Number PCR Array and Assay online data analysis tool

B1. Go to http://pcrdataanalysis.sabiosciences.com/cnv to access the online data analysis tool. Select the real-time PCR cycler used (i.e., Rotor-Gene cycler or Non-Rotor Gene cycler).

B2. Upload the raw C_T data. In the "Load Excel file" field, click the "Browse…" button. In the pop-up window that appears, locate the Excel file containing the C_T data to be analyzed, and then select "array/assay layout", "target assay number", and "replicate reaction number", and click "Open".

Note: The "target assay number" should include the MRef assay.

Note: The input Excel file should follow the format of the "Sample File" provided.

B3. Click the "Upload" button.

The uploaded raw C_T data is displayed.

B4. Assign the "Calibrator Group" if any. Assign sample groups.

B5. Click the "Update" button in the upper left corner.

B6. Click the "QC" button.

B7. If it is necessary to remove a sample that does not pass quality control (indicated on "QC" page as "Inquire"), remove the sample data from the input Excel file, and return to step B2 to reupload the raw C_T data. Alternatively, to keep any sample that does not pass quality control, proceed to step B8.

B8. Click the "Results" button at the top of the page. Click the "Copy Number" button or "p-value" button. Sample copy number calls and statistical significance are displayed in tabular format. Click "Export" to export the results.

Copy number call calculation methods

The real-time PCR for each gene of interest (GOI) is performed at the same time as the PCR for the reference gene (MRef). In theory, the difference between the C_T value of a GOI (C_T^{GOI}) and that of the multi-copy reference assay (C_T^{MRef}) (i.e., $C_T^{GOI} - C_T^{MRef} = \Delta C_T^{GOI}$) is the same across all samples with equivalent

copies of the GOI, even if the amount of DNA used in the PCR varies between samples. Therefore the $\Delta\Delta C_T$ method can be used to calculate the copy number when a ΔC_T^{GOI} value is known for a known copy number of the GOI in a reference genome (e.g., 2 for a normal diploid genome).

There are 2 ways to calculate the $\Delta\Delta C_T$ for a GOI for copy number calls in test samples based upon the 2 types of reference ΔC_T^{GOI} used.

- The calibrator genome method compares test results with the ΔC_T^{GOI} from a calibrator genome (normal diploid genome) ($\Delta C_T^{GOI\text{-}C}$). This method can be used when a calibrator sample is available. The normal diploid/calibrator DNA is tested on the same type of array or assay as used for test samples. The ΔC_T^{GOI} for the calibrator genome, $\Delta C_T^{GOI\text{-}C}$, is calculated as follows.

  $$\Delta C_T^{GOI\text{-}C} = C_T^{GOI\text{-}C} - C_T^{MRef\text{-}C}$$

  For a test sample the $\Delta C_T^{GOI\text{-}T}$ for the gene of interest is

  $$\Delta C_T^{GOI\text{-}T} = C_T^{GOI\text{-}T} - C_T^{MRef\text{-}T}$$

  The $\Delta\Delta C_T^{GOI}$ value is:

  $$\Delta\Delta C_T^{GOI} = \Delta C_T^{GOI\text{-}T} - \Delta C_T^{GOI\text{-}C}$$

  The predicted GOI copy number (CN) in a test sample (assuming the calibrator genome has 2 copies of the GOI) is:

  $$CN_{GOI\text{-}T} = 2 \times 2^{-\Delta\Delta C_T GOI}$$

- The median genome method uses the median ΔC_T^{GOI} ($\Delta C_T^{GOI\text{-}M}$) from a large set of samples (e.g., >20) or a sample set without a calibrator sample. The calculation method is the same as above except that the median ΔC_T^{GOI} from a set of samples ($\Delta C_T^{GOI\text{-}M}$) is used as the calibrator ΔC_T of the GOI. The rationale is that the median C_T^{GOI} of a set of samples will represent a normal diploid genome with 2 copies of the gene (based on the assumption that the frequency for copy gain or loss for a particular locus is lower than 50% in the sample set).

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

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Ordering Information

Product	Contents	Cat. no.
qBiomarker Copy Number PCR Array*	Arrays of assays for real-time PCR-based copy number alteration and variation profiling; available in 96-well, 384-well, and Rotor-Disc formats	Varies
qBiomarker Copy Number PCR Assay (200)*	For 200 reactions; Mix of 2 gene-specific primers provided in solution	337812
qBiomarker SYBR Fluor Mastermix (2)†	For 2 x 96 assays in 96-well plates	337830
qBiomarker SYBR ROX Mastermix (2)†	For 2 x 96 assays in 96-well plates	337820
qBiomarker SYBR ROX FAST Mastermix (2)†	For 2 x 96 assays in 96-well plates	337840
Related products		
DNA QC Plate	Array of assays for evaluating DNA quality for real-time PCR-based arrays; available in 96-well, 384-well, and Rotor-Disc formats	Inquire
Nuclease-Free Water (10 x 50 ml)	10 x 50 ml nuclease-free water prepared without the use of DEPC (diethylpyrocarbonate)	129114
Nuclease-Free Water (1000 ml)	1000 ml nuclease-free water prepared without the use of DEPC (diethylpyrocarbonate)	129115
Nuclease-Free Water (5 liters)	5 liters nuclease-free water prepared without the use of DEPC (diethylpyrocarbonate)	129117
RT² PCR Array Loading Reservoir	12 x 5 ml capacity, irradiation-sterilized loading reservoirs	338162

* QIAGEN reserves the right to occasionally redesign individual qBiomarker Copy Number PCR Assays for improved performance. This revision history can be accessed by contacting Technical Services and supplying the batch numbers from your assays and arrays.

† Larger sizes available; please inquire.

Product	Contents	Cat. no.
384EZLoad Covers (4)	Pack of 4 color-coded covers for loading 384-well plates	338125
DNA purification kits		
QIAamp DNA Mini Kit (50)	For 50 DNA preps: 50 QIAamp Mini Spin Columns, QIAGEN Proteinase K, Reagents, Buffers, Collection Tubes (2 ml)	51304
QIAamp DNA Mini Kit (250)	For 250 DNA preps: 250 QIAamp Mini Spin Columns, QIAGEN Proteinase K, Reagents, Buffers, Collection Tubes (2 ml)	51306
DNeasy Blood & Tissue Kit (50)	For 50 DNA preps: 50 DNeasy Mini Spin Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	69504
DNeasy Blood & Tissue Kit (250)	For 250 DNA preps: 250 DNeasy Mini Spin Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	69506
QIAamp DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute® Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	56404
RNase A (17,500 U)	2.5 ml (100 mg/ml; 7000 units/ml, solution)	19101

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www.qiagen.com

Australia ■ techservice-au@qiagen.com

Austria ■ techservice-at@qiagen.com

Belgium ■ techservice-bnl@qiagen.com

Brazil ■ suportetecnico.brasil@qiagen.com

Canada ■ techservice-ca@qiagen.com

China ■ techservice-cn@qiagen.com

Denmark ■ techservice-nordic@qiagen.com

Finland ■ techservice-nordic@qiagen.com

France ■ techservice-fr@qiagen.com

Germany ■ techservice-de@qiagen.com

Hong Kong ■ techservice-hk@qiagen.com

India ■ techservice-india@qiagen.com

Ireland ■ techservice-uk@qiagen.com

Italy ■ techservice-it@qiagen.com

Japan ■ techservice-jp@qiagen.com

Korea (South) ■ techservice-kr@qiagen.com

Luxembourg ■ techservice-bnl@qiagen.com

Mexico ■ techservice-mx@qiagen.com

The Netherlands ■ techservice-bnl@qiagen.com

Norway ■ techservice-nordic@qiagen.com

Singapore ■ techservice-sg@qiagen.com

Sweden ■ techservice-nordic@qiagen.com

Switzerland ■ techservice-ch@qiagen.com

UK ■ techservice-uk@qiagen.com

USA ■ techservice-us@qiagen.com

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